Selective Fluorescence Detection of Cholesterol Using an Anthracene-Triazole Probe: A Turn-On, Enzyme/Metal-Free Approach

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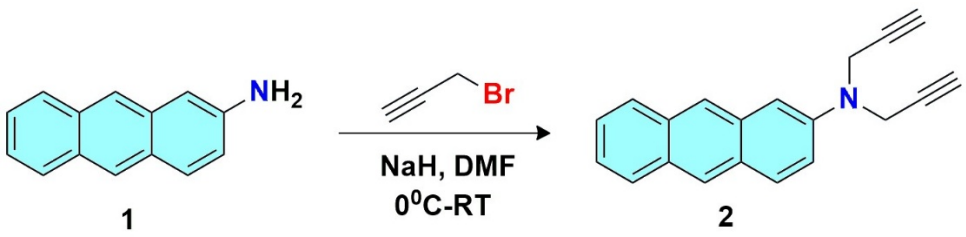
1. Materials and methods

All the chemicals were purchased from Sigma Aldrich, Alfa Aeser, Spectrochem, Merck and TCI and used without further purification. LC-MS experiments were carried out on a Shimadzu LC-MS-8045 with a Sprite TARGA C18 column (40 × 2.1 mm, 5 µm) monitoring at 210 nm and 254 nm with positive mode for mass detection. Solvents for LC-MS were water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). Compounds were eluted at a flow rate of 0.5 mL/min with a gradient of 5% solvent B for 2 min, then linearly from 5% to 40% solvent B over 4 min, then from 40% to 60% for 10 min, and lastly, it was brought down to 5% solvent B in 2 min, and then the procedure proceeded till for 2 min before stopping. Before injecting the sample, the column was washed twice, once with 50% Solvent B and once with 95% Solvent B. ^{1}H NMR spectra were recorded on Bruker AV III 400 MHz. The data were analyzed by MestReNova (version 8.1.1). ^{1}H NMR shifts are reported in units of ppm relative to tetramethyl silane. The data are presented in the following order: chemical shift, peak multiplicity (s=singlet, d=doublet, t=triplet, m=multiplet) and proton number. Fluorescence was recorded on Perkin Elmer FL 6500. The absorption and emission spectra were plotted in OriginPro 8.5.1.

2. Synthesis

2.1 Synthesis of anthracene alkyne (**2**)

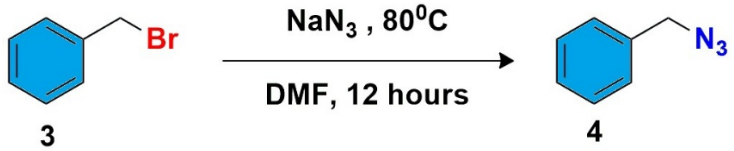
To a solution of commercially available 2-aminoanthracene (250 mg, 1.293 mmol, 1 equiv.) in 500 µL of N, N′-dimethylformamide (DMF), cooled in an ice bath for 5-10 minutes under magnetic stirring, sodium hydride (310 mg, 12.935 mmol, 10 equiv.) was added portion-wise over 5-10 minutes. Subsequently, propargyl bromide (980 µL, 12.935 mmol, 10 equiv.) was added dropwise. The reaction mixture was stirred at room temperature for 4 hours. Completion of the reaction was monitored by TLC. The reaction was quenched with water and extracted with ethyl acetate. Product **2** was isolated as yellow solid after silica column chromatography. The reaction yield was 86% (299 mg). MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{20}H_{15}N$ 270.12; Found 270.25. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.30 (s, 1H), 8.25 (s, 1H), 7.94 – 7.91 (m, 3H), 7.43 – 7.35 (m, 2H), 7.30 (dd, J = 5.0, 2.3 Hz, 2H), 4.27 (d, J = 2.4 Hz, 4H), 2.29 (t, J = 2.3 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 144.92 (s), 132.63 (s), 132.37 (s), 130.43 (s), 129.49 (s), 128.24 (s), 127.90 (s), 127.68 (s), 125.97 (s), 125.43 (s), 124.41 (s), 124.19 (s), 119.80 (s), 109.40 (s), 79.05 (s), 73.06 (s), 40.82 (s).



Scheme S1. Synthesis of **2**

2.2 Synthesis of benzyl azide (**4**)

Benzyl bromide (348 µL, 2.923 mmol, 1.0 equiv.) was added to a 100 mL round-bottom flask, followed by sodium azide (228 mg, 3.507 mmol, 1.2 equiv.) and 1 mL of N, N-dimethylformamide (DMF). The reaction mixture was refluxed at 80 °C for 12 hours. Product formation was indicated by a spot visible in an iodine chamber. Upon completion, the reaction was quenched with water and the mixture was extracted with ethyl acetate. Product **4** was isolated as liquid after silica column chromatography. The reaction yield was 74% (288 mg). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 - 7.32 (m, 5H), 4.34 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 128.86 (s), 128.28 (d, J = 9.4 Hz), 54.76 (s).

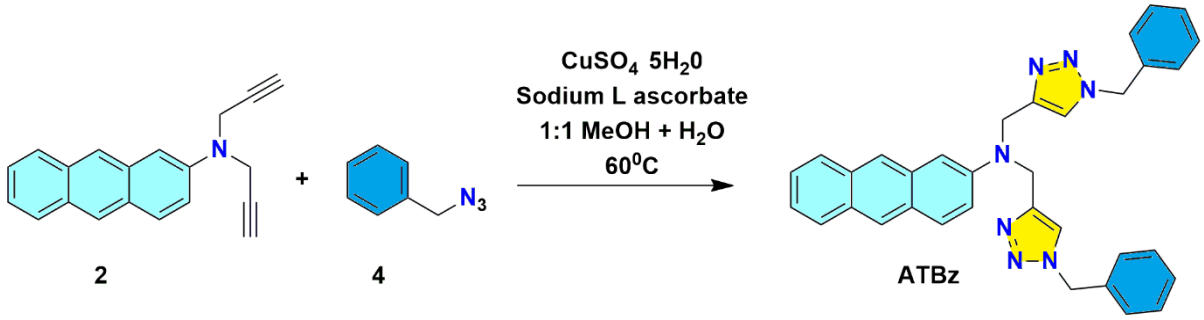


Scheme S1. Synthesis of **4**

2.3 Synthesis of **ATBz**

To the solution of compound **2** (200 mg, 0.7435 mmol, 1.0 equiv.), **4** (98.87 mg, 1.4870 mmol, 2.1 equiv.) was added. Sodium L-ascorbate (1.3 mg, 0.0074 mmol, 0.01 equiv.) and copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, 9.2 mg, 0.037 mmol, 0.05 equiv.) were then added as catalysts. A 1:1 mixture of methanol and water was used as the solvent, and the reaction mixture was stirred in an oil bath at 60 °C for 1 hour. Reaction progress was monitored by TLC. Upon completion, methanol was evaporated, and the mixture was filtered to remove the solid sodium L-ascorbate. The filtrate was then extracted with ethyl acetate and water. The pure product **ATBz** was obtained as a pale-yellow solid after column chromatography. The reaction yield was 70 % (278 mg). MS (ESI) m/z: $[M+H]^+$ Calcd for $C_{34}H_{29}N_7$ 536.25; Found 536.25. ^{1}H

NMR (400 MHz, $CDCl_3$) δ 8.24 (s, 1H), 8.08 (s, 1H), 7.92 – 7.83 (m, 3H), 7.42 – 7.33 (m, 3H), 7.32 (s, 2H), 7.29 – 7.26 (m, 6H), 7.15 (dd, *J* = 3.9, 2.0 Hz, 4H), 7.08 (d, *J* = 2.3 Hz, 1H), 5.41 (s, 4H), 4.74 (s, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 145.50 (s), 145.04 (s), 134.62 (s), 132.90 (s), 132.40 (s), 129.98 (s), 129.54 (s), 129.06 (s), 128.65 (s), 128.21 (s), 127.82 (s), 127.60 (s), 127.20 (s), 125.93 (s), 124.00 (s), 123.35 (s), 122.16 (s), 118.94 (s), 106.81 (s), 54.32 – 54.13 (m), 46.93 (s).



Scheme S1. Synthesis of **ATBz**

3. Mass Spectra

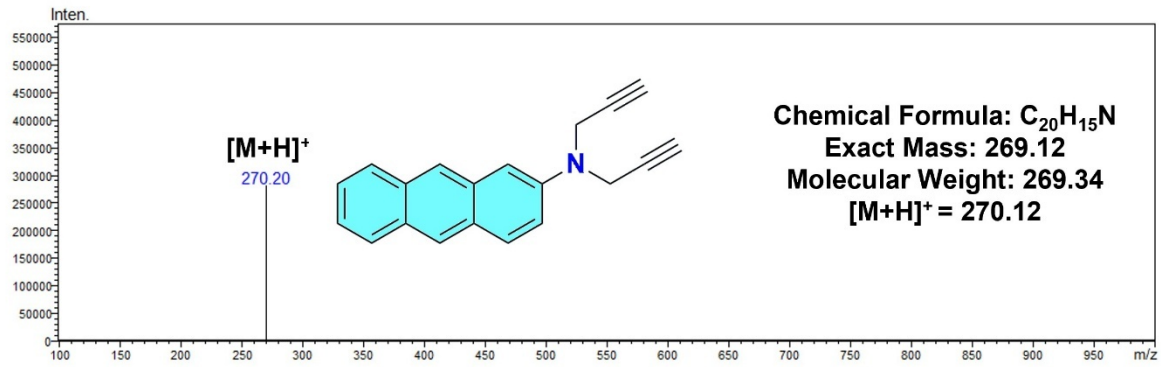


Figure S1. Mass spectrum of compound **2**.

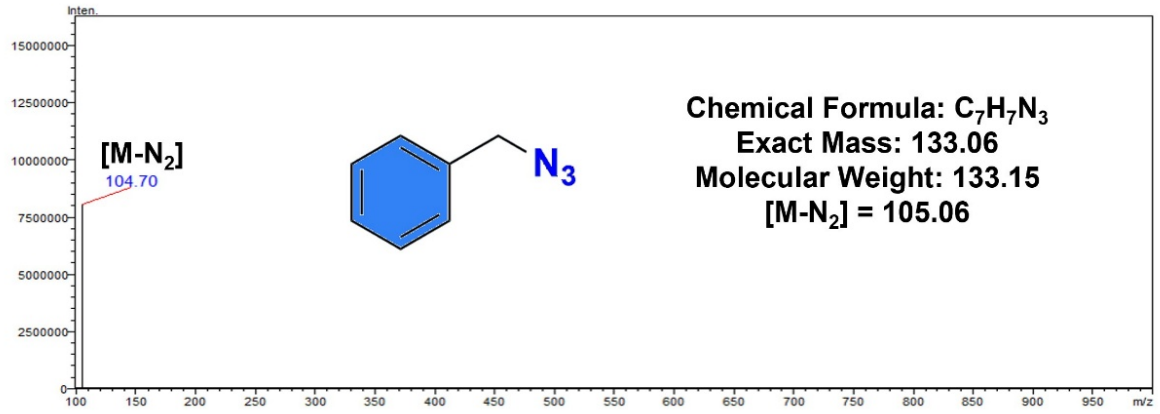


Figure S2. Mass spectrum of compound **4**.

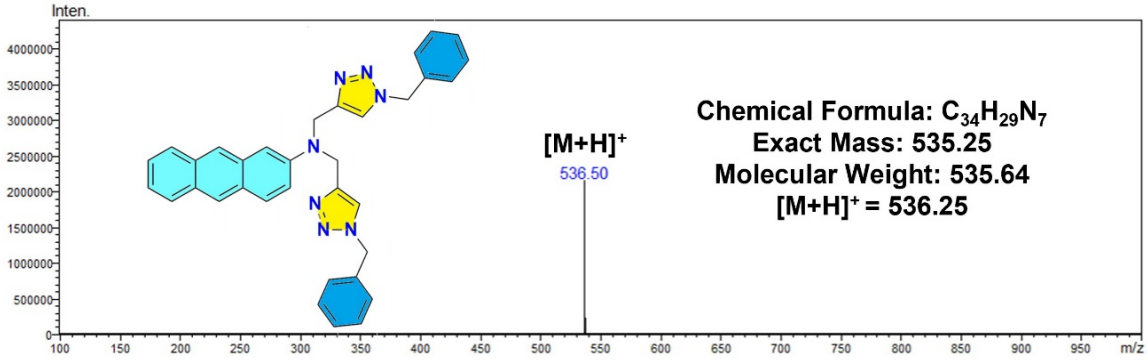


Figure S3. Mass spectrum of compound **ATBz**.

4. FT-IR Spectra

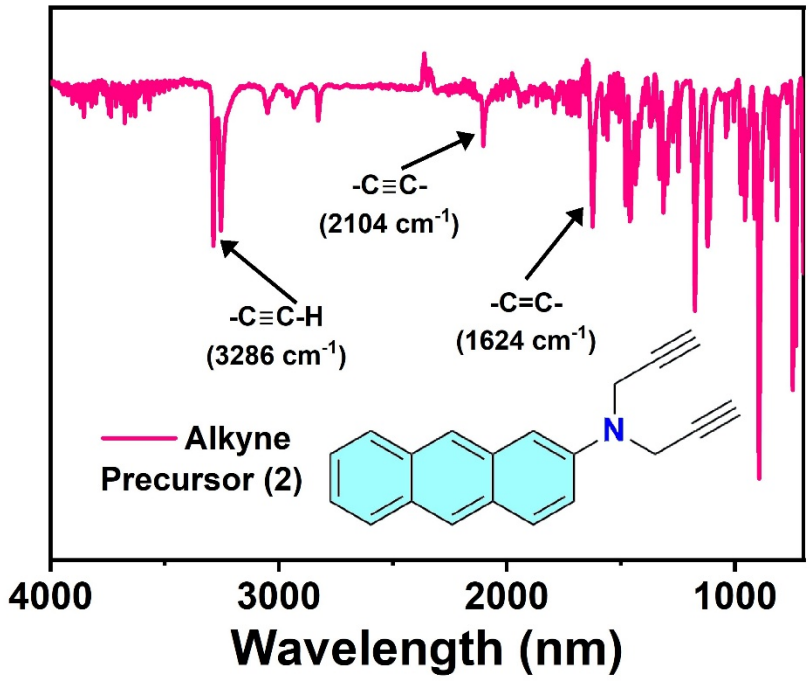


Figure S4. FT-IR spectrum of compound **2**.

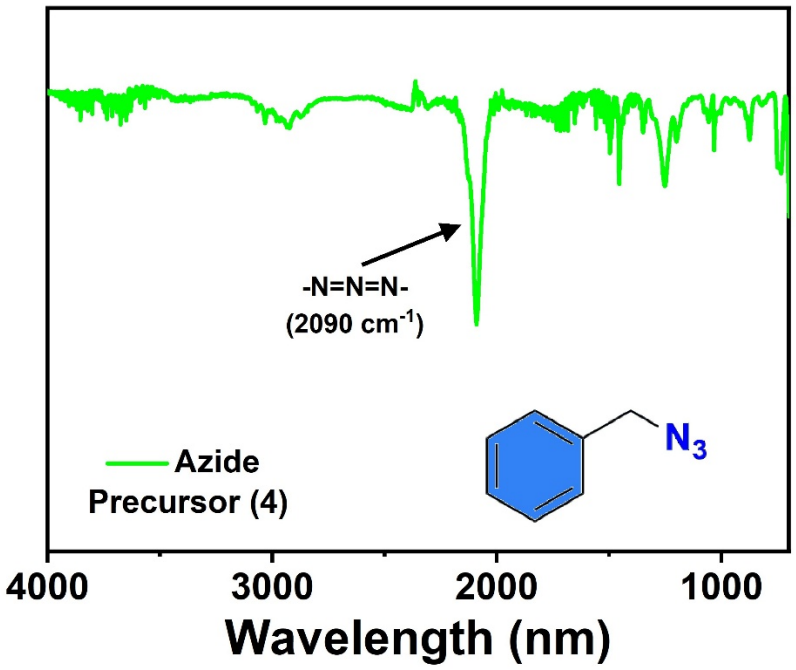


Figure S5. FT-IR spectrum of compound **4**.

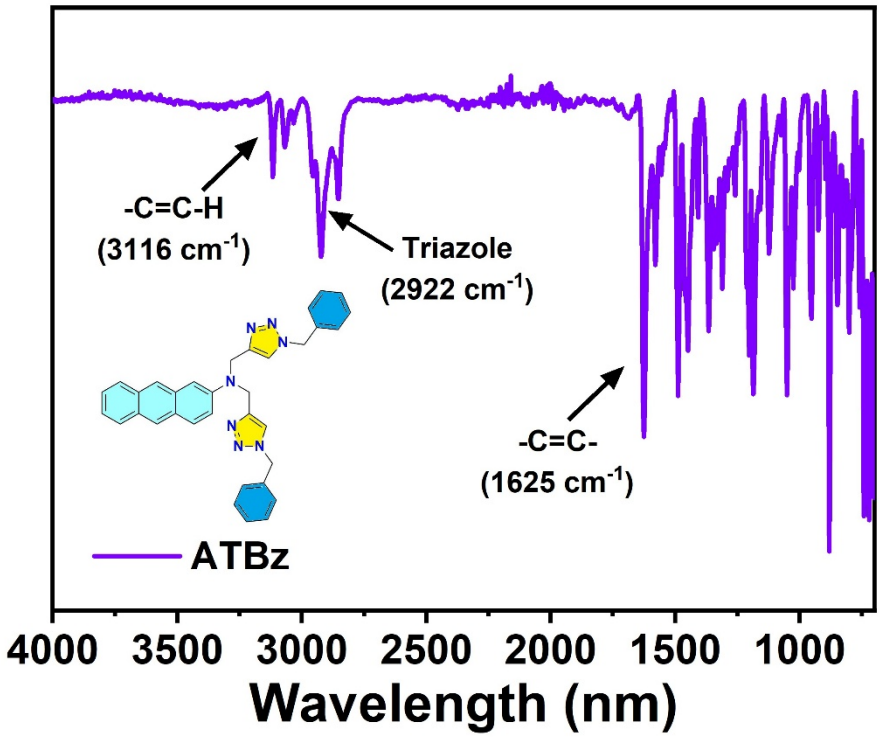


Figure S6. FT-IR spectrum of compound **ATBz**.

5. NMR Spectra

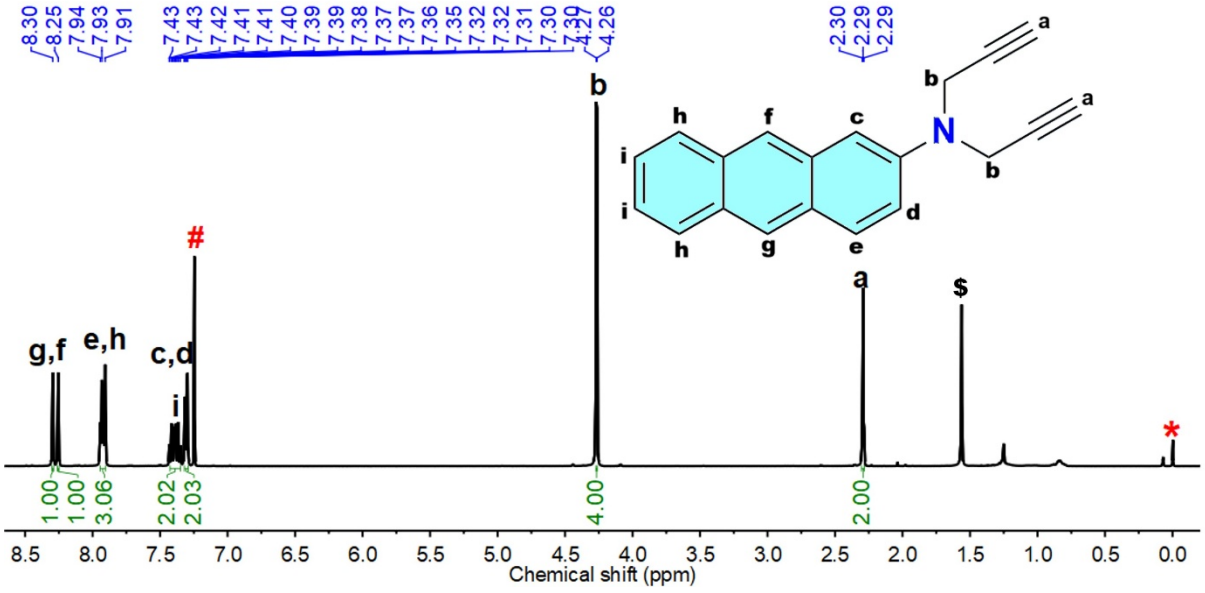


Figure S7. ^{1}H NMR spectrum of **2** recorded at 400 MHz in $CDCl_3$ at 298 K. Signals marked with (*) and (#) denote the residual proton of internal standard tetramethyl silane and $CDCl_3$ and ($) represents the water peak respectively.

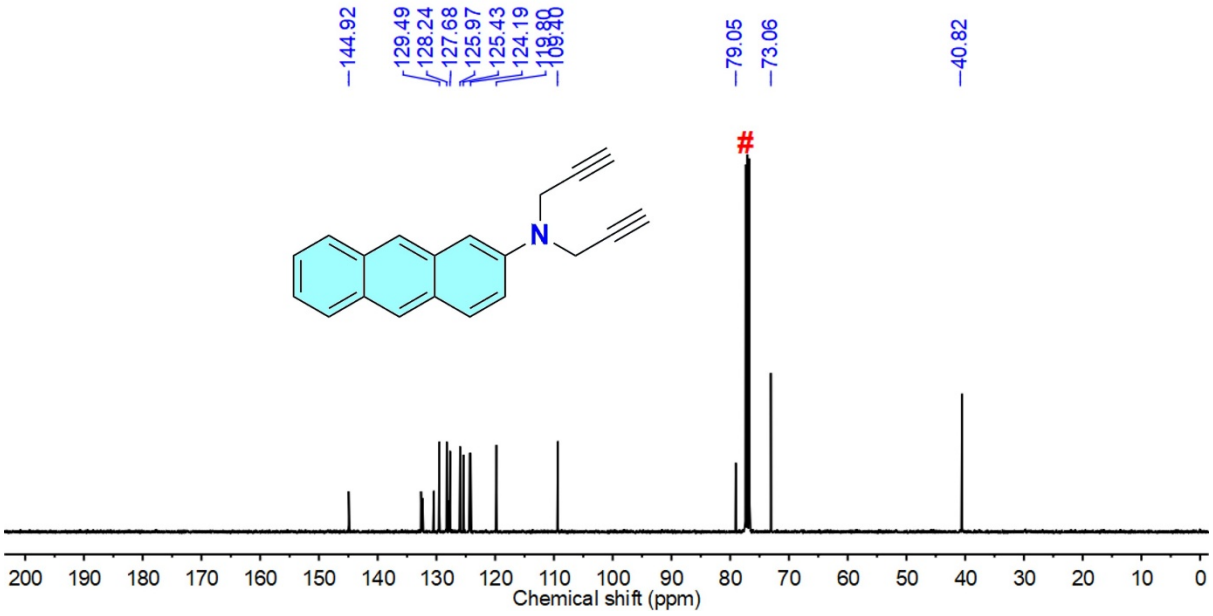


Figure S8. ^{13}C NMR spectrum of **2** recorded at 101 MHz in $CDCl_3$ at 298 K. Signal marked with (#) denotes residual carbon of $CDCl_3$.

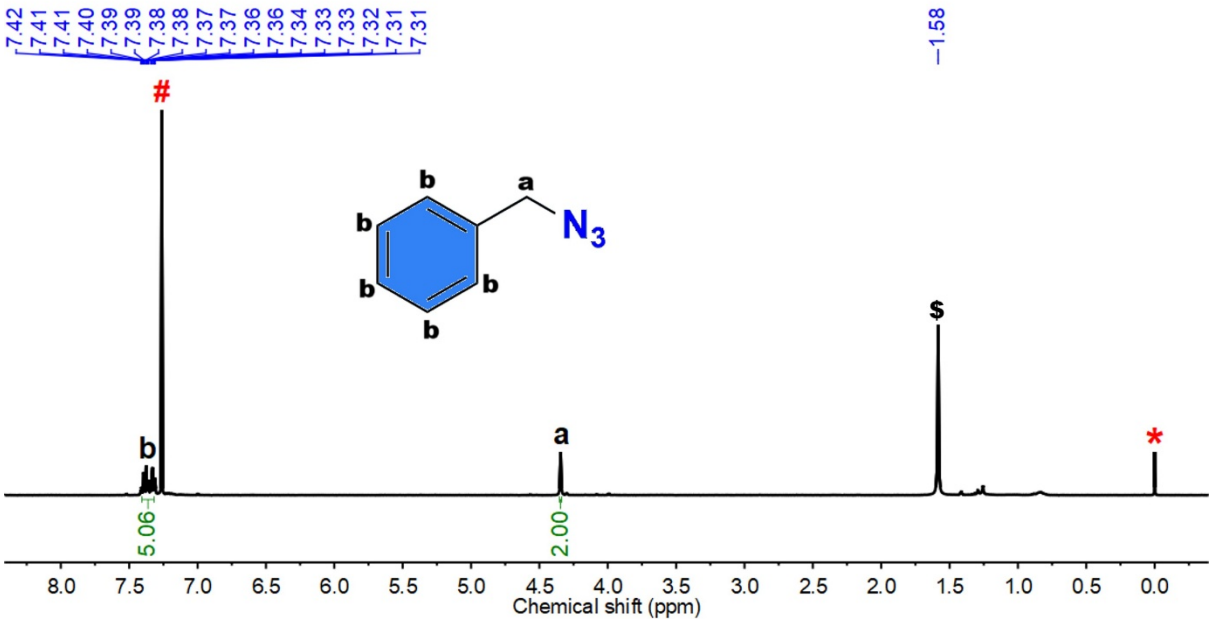


Figure S9. ^{1}H NMR spectrum of **4** recorded at 400 MHz in $CDCl_3$ at 298 K. Signals marked with (*) and (#) denote the residual proton of internal standard tetramethyl silane and $CDCl_3$ and ($) represent the water peak respectively.

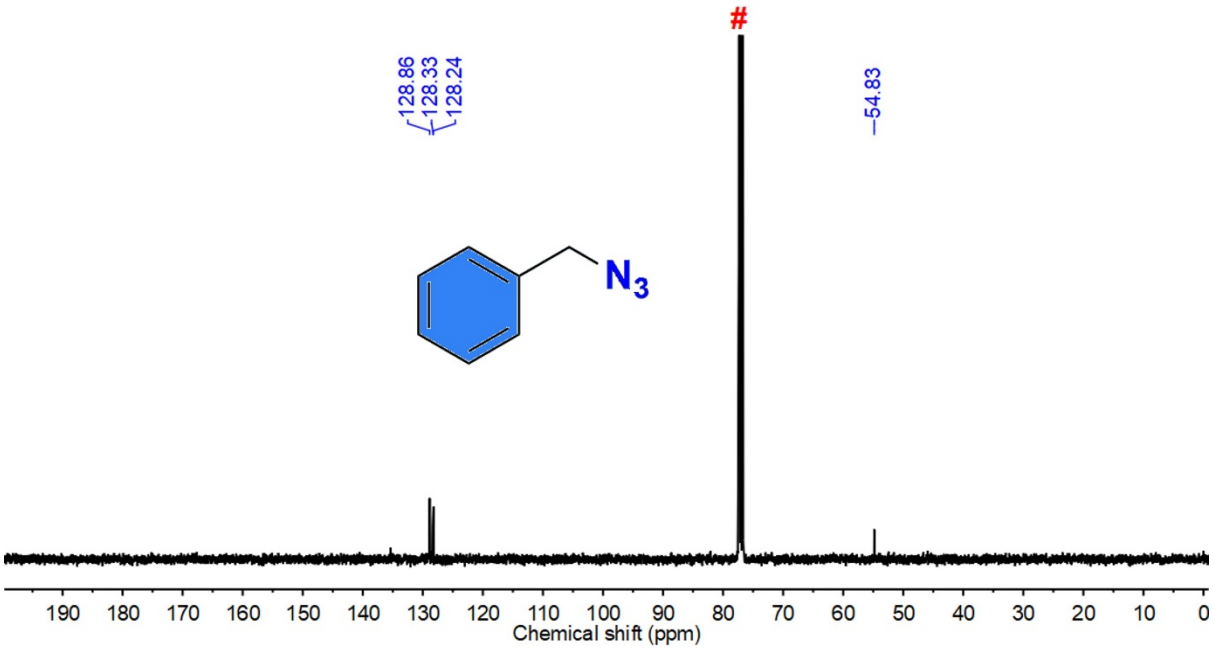


Figure S10. ^{13}C NMR spectrum of **4** recorded at 101 MHz in $CDCl_3$ at 298 K. Signal marked with (#) denotes residual carbon of $CDCl_3$.

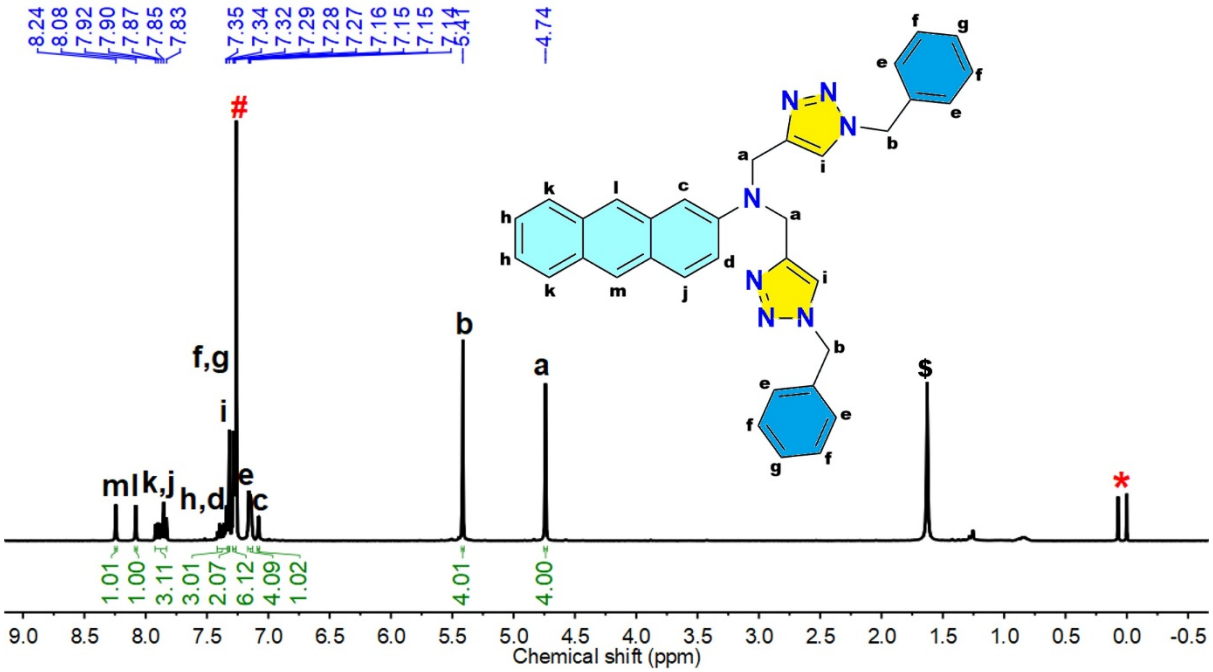


Figure S11. ^{1}H NMR spectrum of **ATBz** recorded at 400 MHz in $CDCl_3$ at 298 K. Signals marked with (*) and (#) denote the residual proton of internal standard tetramethyl silane and $CDCl_3$ and ($) represent the water peak respectively.

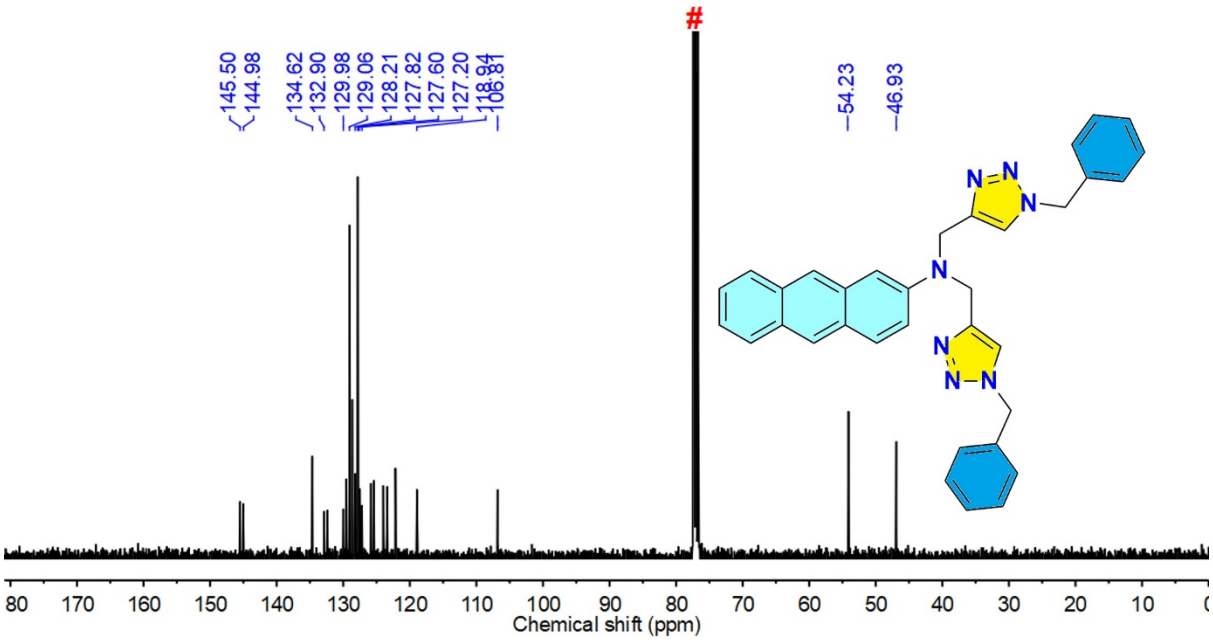


Figure S12. ^{13}C NMR spectrum of **ATBz** recorded at 101 MHz in $CDCl_3$ at 298 K. Signal marked with (#) denotes residual carbon of $CDCl_3$.

6. PXRD Analysis

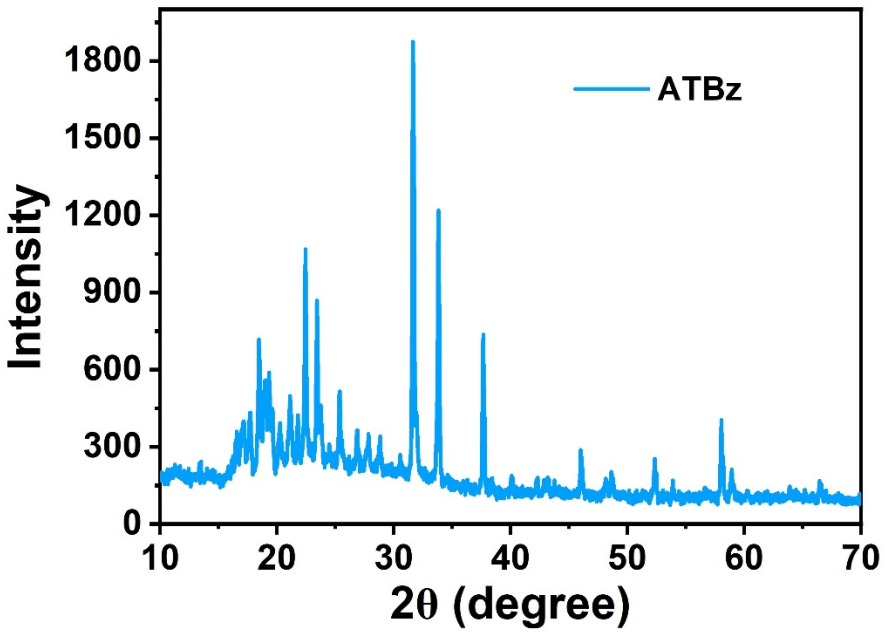


Figure S13. The PXRD pattern of **ATBz**

Table S1: Various known cholesterol sensors reported in the literature

Enzymatic detection of cholesterol

Sl. No.	Journal	LOD	Response	Selectivity	Linear range	Response time
1	Nanoscale, **2025**, *17*, 10043-10056	3 μM	'Turn on'	Non selective, detect both cholesterol and glucose	2–60 μM	-
2	Microchem. J. **2025**, *208*, 112618	0.014 mM	Ratiometric signal	Selective to cholesterol	0.05-1.0 mM	5 min
3	ACS Appl. Nano Mater. **2021**, *4*, 13612-13624	4.89 nM	'Turn on'	non selective, detect biothiols, Acetyl cholinester ase, and Chlorpyrifos	10 to 200 nM	-
4	ACS Appl. Nano Mater. **2021**, *4*, 9132– 9142	0.092 μM	'Turn on'	Non selective, Detect glucose and cholesterol	0.5 to 250 μM	-
5	ACS Omega **2019**, *4*, 9333-9342	3.6 nM	ratiometric fluorescence signal	Non selective, Detect cholesterol and uric acid	0.01 μM to 150 μM.	25 min

Metal organic framework-based cholesterol detection

Sl. No.	Journal	LOD	Response	Selectivity	Linear range	Response time
1	Microchim. Acta, **2022**, *189*, 1–10	0.073 μM	'Turn off'	Selective to cholesterol	0.1–2.4 μM	15 min
2	Nano., **2021,** 32, 315502	1.57 μM	'Turn on'	Selective to cholesterol	10–360 μM	-
3	Microchem. J., **2021**, *164*, 106001	0.01 μM	'Turn on'	Selective to cholesterol	0.04–1.60 μM	10 min
4	J. Colloid Interface Sci., **2023**, *649*, 601–615	0.38 μM	'Turn on'	Selective to cholesterol	2–160 μM	25 min
5	J. Mater. Chem. C, **2019**, *7*, 12674–12681	0.40 nM	'Turn on'	Selective to cholesterol	0–180 μM	30 min

Nanoparticle based cholesterol detection

Sl. No.	Journal	LOD	Response	Selectivity	Linear range	Response time
1	Anal. Chem., **2015**, *87*, 10362–10367	1.4 μM	'Turn off'	-	1–100 μM	-
2	Chemistry Select, **2019**, *4*, 14222–14227	0.204 μM	'Turn off'	Selective to cholesterol	0.5–40 μM	-
3	Green Chem., **2016**, *18*, 4245–4253	56 μM	'Turn on'	Selective to cholesterol	0–800 μM	≤5 minutes
4	Talanta, **2023**, *253*, 123908	186 μM	'Turn on'	Selective to cholesterol	400–5170 μM	Quick response
5	Anal. Bioanal. Chem., **2022**, *414*, 3827–3836	0.018 μM	Turn off'	Selective to cholesterol	0.025–10 μM	40 min

Small molecule-based cholesterol detection

Sl. No.	Journal	LOD	Response	Selectivity	Linear range	Response time
1	Chem. Commun., **2023**, *59*, 1501-1504.	4 nM	'Turn on'	Selective to cholesterol	0-8 mM	Quick response
2	Bioorg. Chem., **2025**, *160,* 108483	10 nM	'Turn on'	Selective to cholesterol	0.02-0.6 eq. of cholesterol	Quick response
3	**This work**	**100 nM**	**'Turn on'**	**Selective to cholesterol**	**0- 7 mM**	**Quick response**

7. Photophysical studies

7.1 UV- Visible absorption emission spectra

A 20 µM solution of **ATBz** was prepared in various solvents, including toluene, dichloromethane (DCM), acetonitrile (ACN), and dimethyl sulfoxide (DMSO). UV-vis absorption and fluorescence emission spectra were recorded separately for each solution. The absorption maxima were observed at 296 nm (toluene), 300 nm (DCM), 302 nm (ACN), and 306 nm (DMSO). Correspondingly, the emission spectra showed peaks at 469 nm, 480 nm, 489 nm, and 500 nm, respectively. All spectroscopic measurements were carried out at a constant temperature of 25 °C.

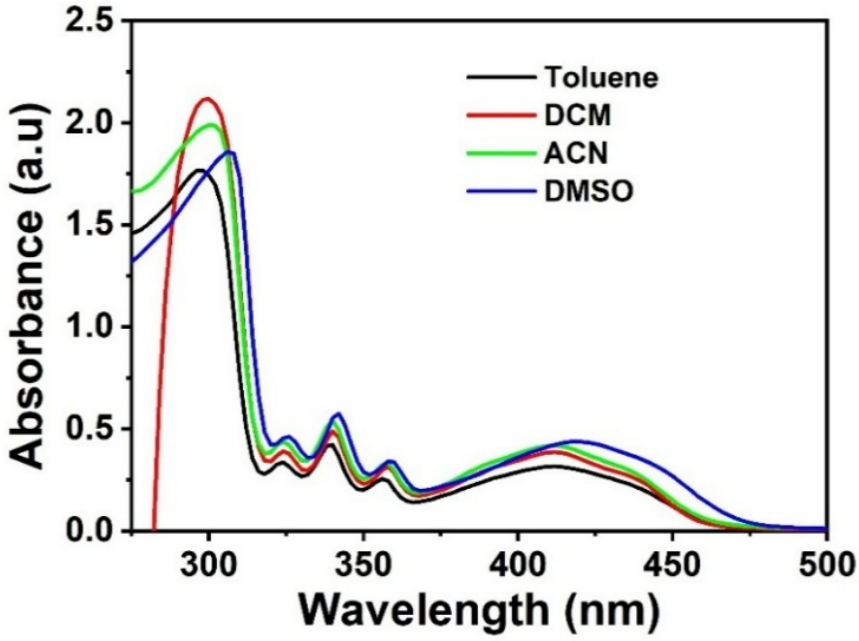


Figure S14. The absorption spectra of **ATBz** (20 μM) in toluene, dichloromethane (DCM), acetonitrile (ACN), and dimethyl sulfoxide (DMSO) at 25^0C.

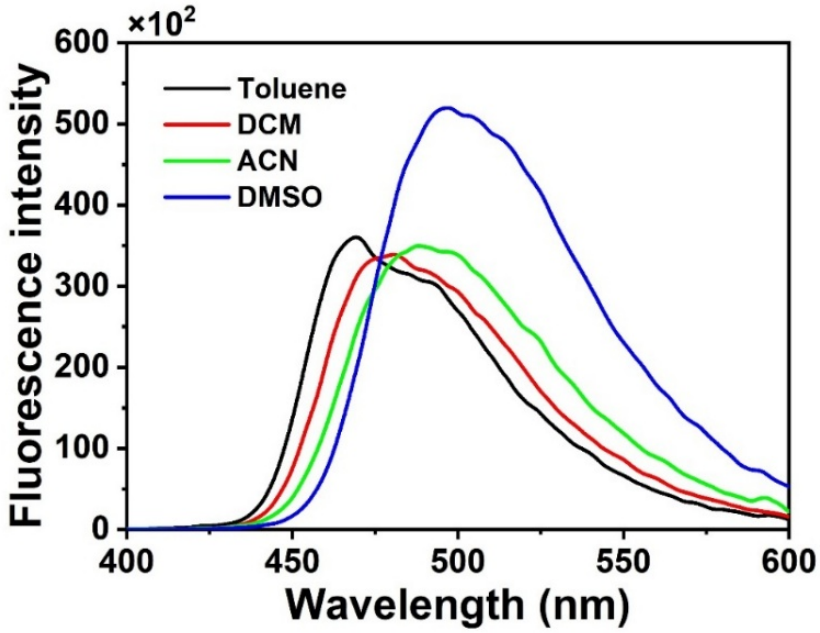


Figure S15. The emission spectra of **ATBz** (20 μM) in toluene, dichloromethane (DCM), acetonitrile (ACN), and dimethyl sulfoxide (DMSO) at 25^0C when excited at 296 nm.

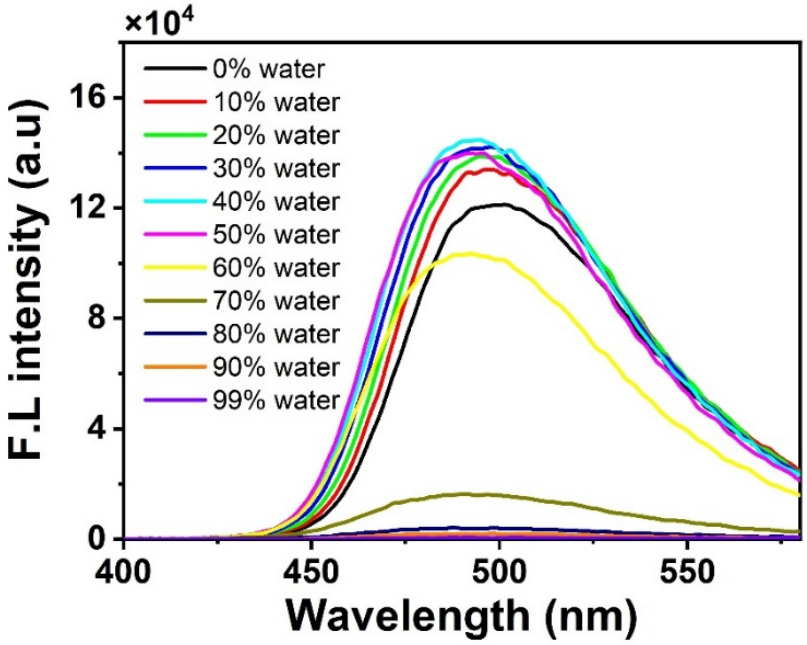


Figure S16. The fluorescence spectra **ATBz** (20 μM) upon varying % volume fraction of water in DMSO (0 to 99, v/v) at 25^0C when excited at 296 nm.

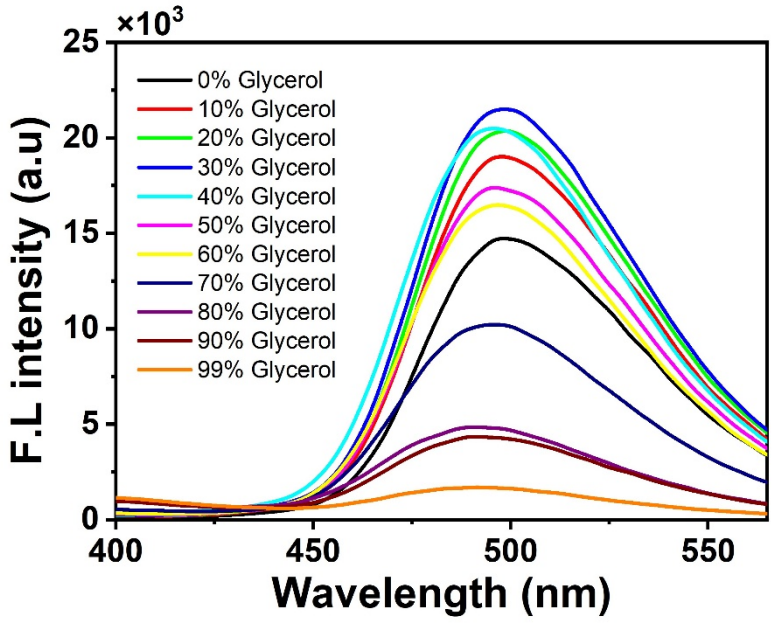


Figure S17. The fluorescence spectra **ATBz** (20 μM) upon varying % volume fraction of glycerol in DMSO (0 to 99, v/v) at 25^0C when excited at 296 nm.

7.2 Quantum yield calculation

The fluorescence quantum yield of the compound **ATBz** was determined in various solvents, including toluene, dichloromethane, tetrahydrofuran, acetonitrile, methanol, dimethyl sulfoxide, and a water/DMSO mixture, using the following equations.

For different solvent systems,

$$Q_S = Q_R \frac{I_S}{I_R} \frac{A_R}{A_S} \frac{\eta_S^2}{\eta_R^2}$$

For different fraction of water/DMSO systems,

$$Q_S = Q_R \frac{I_S}{I_R} \frac{A_R}{A_S} \frac{\eta_{mix}^2}{\eta_R^2}$$

Where:

Q_S and Q_R represent the quantum yield of the sample **ATBz** and the standard reference quinine sulphate.

I_S and I_R denote the integrated areas under the emission spectra for the sample and reference.

A_S and A_R correspond to the absorbance of the sample and reference.

η_S, η_R, and η_{mix} represent the refractive indices of the solvent used for the sample, the reference, and the mixed solvent system, respectively.

Table S2. Quantum yield of **ATBz** in different solvents

Solvent	Quantum yield (Φ)
Toluene	0.47
Dichloromethane	0.59
Tetrahydrofuran	0.48
Acetonitrile	0.54
Methanol	0.57
Dimethyl sulfoxide	0.66

Table S3. Quantum yield of **ATBz** in water/DMSO mixture.

Water/DMSO (%)	Quantum yield (Φ)
0	0.65
20	0.66
40	0.69
60	0.62
80	0.25

Table S4. Quantum yield of **ATBz** in Glycerol/DMSO mixture.

Glycerol/DMSO (%)	Quantum yield (Φ)
0	0.64
20	0.67
40	0.65
60	0.59
80	0.21

8. Cholesterol detection

8.1 Selectivity studies

A 2 mM stock solution of **ATBz** was prepared using DMSO. This stock was diluted with 2 mL of water to create 10 µM sensor solutions, which were then transferred into cuvettes for fluorescence analysis. Additionally, a 5 mM stock solution of cholesterol was prepared in ethanol. Stock solutions of various analytes including arginine, cysteine, glutamic acid, histidine, phenylalanine, serine, tryptophan, valine, ascorbic acid, $CaSO_4$, creatinine, dopamine, glucose, KCl, $MgSO_4$, NaCl, sucrose, urea, uric acid, and cholesterol were also prepared at a concentration of 5 mM in water. To each 10 µM sensor solution, 50 µM of the corresponding analyte from these stock solutions was added, and the fluorescence spectra were

recorded. For the photograph, a solution containing 10 μM of **ATBz** and 100 μM of each analyte was used.

8.2 Absorption spectra of upon the addition of cholesterol

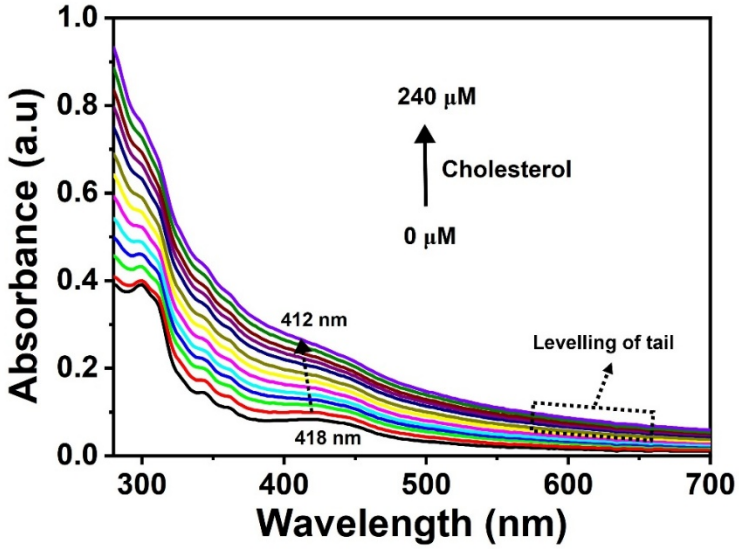


Figure S18: The absorption spectra of **ATBz** (20 μM) upon addition of cholesterol (0-240 μM) in 1% DMSO in water.

8.3 Limit of detection (LOD)

The detection limit of cholesterol was determined using the **3σ/k** method, where **σ** represents the standard deviation and **k** refers to the slope of the plot of fluorescence intensity of **ATBz** against cholesterol concentration. For the fluorescence titration, 5 μM of **ATBz** solution was placed in a cuvette and diluted to a final volume of 2 mL with water. The fluorescence spectrum was recorded. Subsequently, incremental additions of cholesterol solution ranging from 0.1 to 20 μM were made to the same cuvette, and the fluorescence spectra were recorded after each addition. The calibration curve was subsequently replotted using ΔI ($I - I_{blank}$), where I and I_{blank} correspond to the fluorescence intensities in the presence and absence of cholesterol, respectively. A slope of 4.4622×10^9 with an R^2 value of 0.998 was obtained, and the LOD was determined to be (100 ± 1.4) nM.

8.4 Benesi-Hildebrand plot- Determination of association constant K_a

The binding constant (K_a) of **ATBz** and cholesterol was calculated using the following Benesi-Hildebrand equation:

$$\frac{1}{\Delta I} = \frac{1}{\Delta I_{max}} + \left(\frac{1}{K_a[C]}\right)\left(\frac{1}{\Delta I_{max}}\right)$$

Where ΔI is defined as the difference between the emission intensity of **ATBz** at a given cholesterol concentration (**I**) and the emission intensity of **ATBz** in the absence of cholesterol (I_{min}), that is, $\Delta I = I - I_{min}$. Similarly, ΔI_{max} represents the difference between the emission intensity of **ATBz** at full saturation with cholesterol (I_{max}) and the emission intensity of **ATBz** in the absence of cholesterol (I_{min}), expressed as $\Delta I_{max} = I_{max} - I_{min}$. Here, K_a denotes the binding constant, and [C] represents the cholesterol concentration. The value of K_a is determined from the slope and intercept of the plot of $[1 / (I - I_{min})]$ versus **1/[C]**. From the graph, a slope of $1.574 \times 10^{-10} \pm 2.63 \times 10^{-12}$ and an intercept of $1.651 \times 10^{-5} \pm 8.23 \times 10^{-6}$ was obtained.

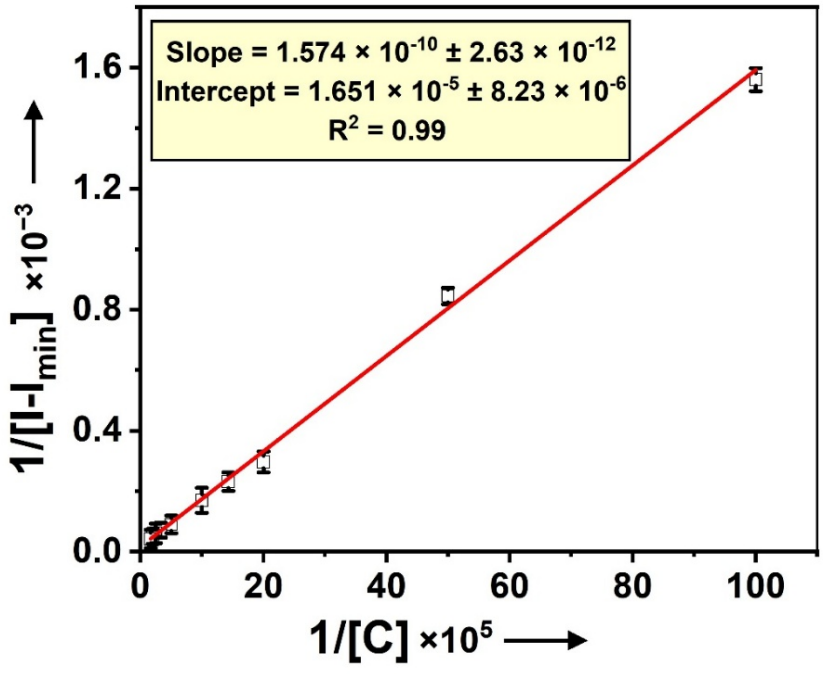


Figure S19. Benesi-Hildebrand plot of **ATBz** and cholesterol in water (0.5 % DMSO) recorded ate 25^0C when excited at 296 nm.

8.5 Competivity studies

A 10 μM solution of **ATBz** in water containing 0.5% DMSO was prepared in a cuvette, and its fluorescence spectra were recorded. Subsequently, 5 mM of each of the other analytes were added individually to the same solution, and the spectra were recorded after each addition. Finally, an equivalent concentration of cholesterol (50 μM) was added to the same solution, and the corresponding emission spectra were recorded. Bar diagrams were constructed by plotting the ratio $\mathbf{I_0/I}$, where $\mathbf{I_0}$ represents the fluorescence intensity of **ATBz** in the absence of an analyte and **I** represents the fluorescence intensity in the presence of the analyte.

8.7 Photograph of ATBz at different cholesterol concentrations under UV lamp

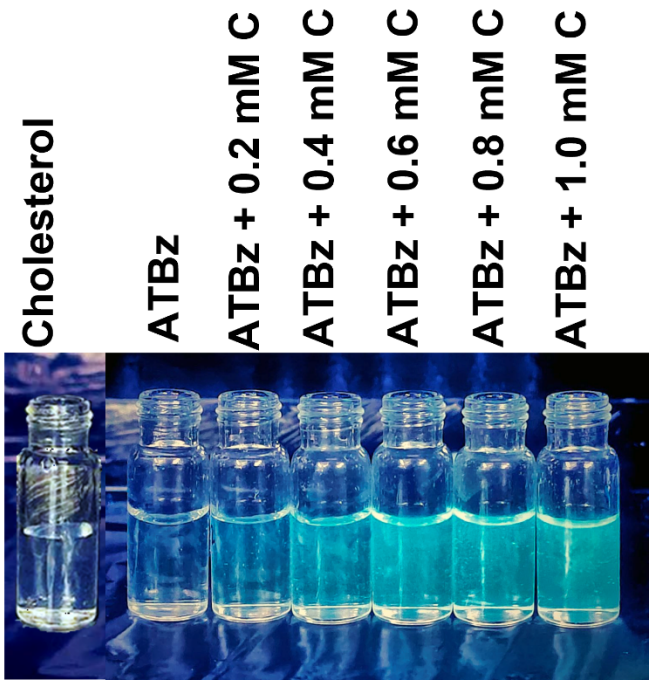


Figure S20. Photograph of **ATBz** (20 μM) under UV lamp at different concentrations of cholesterol.

8.6 pH stability of the sensor system

To prepare the required pH solutions, appropriate ratios of 1.0 M HCl and 1.0 M NaOH were mixed to obtain buffers with pH values from 2 to 10. The fluorescence intensity of a 10 µM **ATBz** solution, with and without 50 µM cholesterol, was then recorded immediately after preparation at each pH.

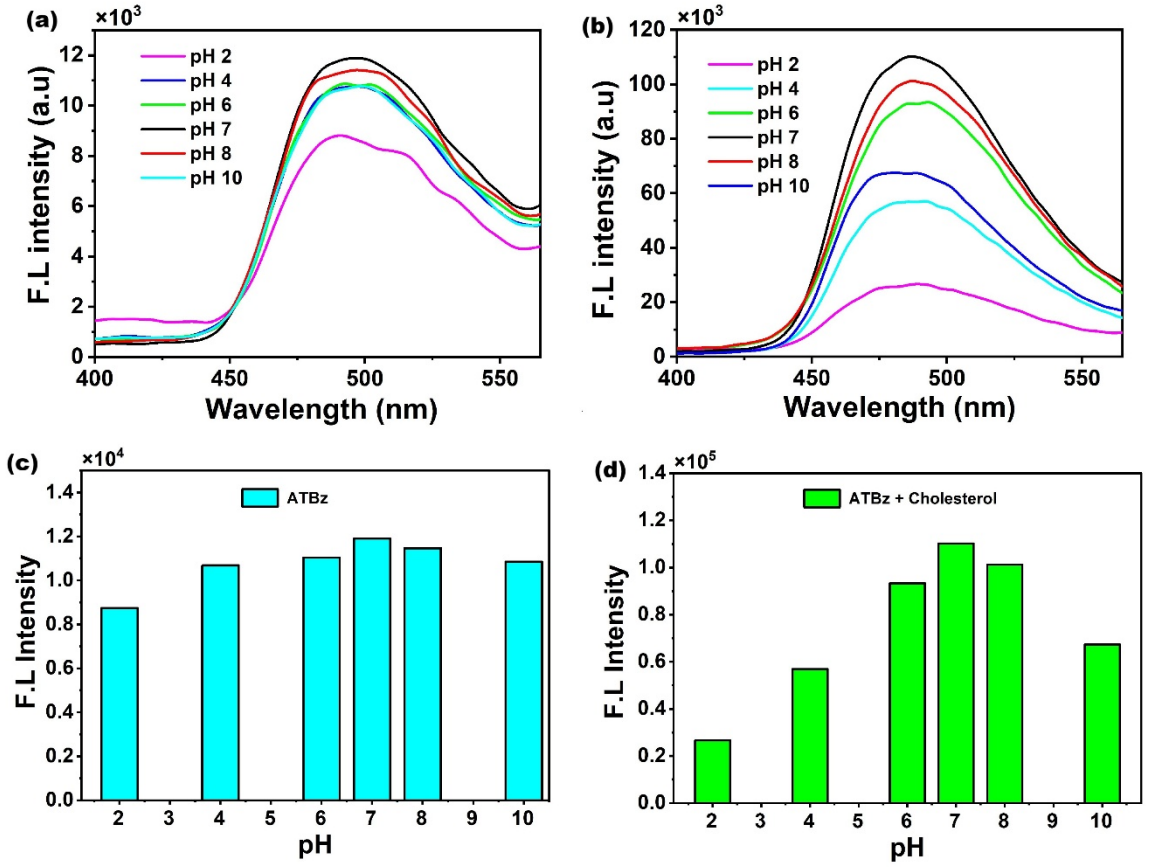


Figure S21. Fluorescence spectra of (a) **ATBz** (10 µM), (b) **ATBz** + cholesterol and corresponding bar diagram of (c) **ATBz** (d) of **ATBz** + cholesterol (50 µM) measured across a pH range of 0-10.

8.8 Dynamic Light Scattering (DLS) analysis

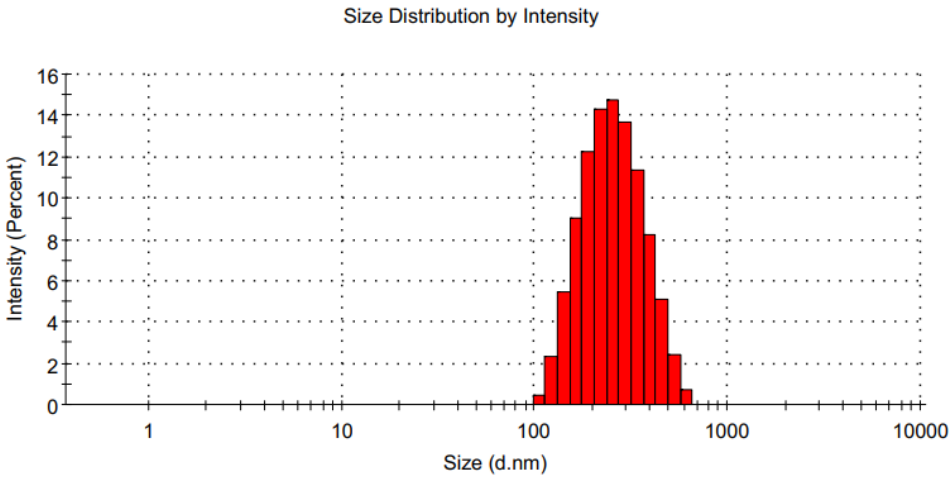


Figure S22. The DLS studies of **ATBz** (10 µM) showing particle size 239 nm upon addition of cholesterol (50 µM) in (0.5 % DMSO) with PDI: 0.134.

8.9 Tyndall effect of ATBz + Cholesterol

The solution of **ATBz** with cholesterol exhibited a distinct Tyndall effect, indicating the formation of nanoscale aggregates.

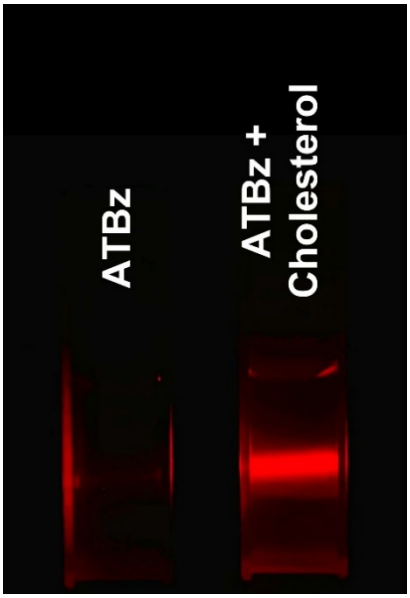


Figure S23. Photographic image of the solution of **ATBz** (10 µM) alone (left) and **ATBz** showing Tyndall effect with the addition of 50 µM of cholesterol solution (right).

8.10 Field Emission Scanning Electron Microscopy (FESEM) analysis

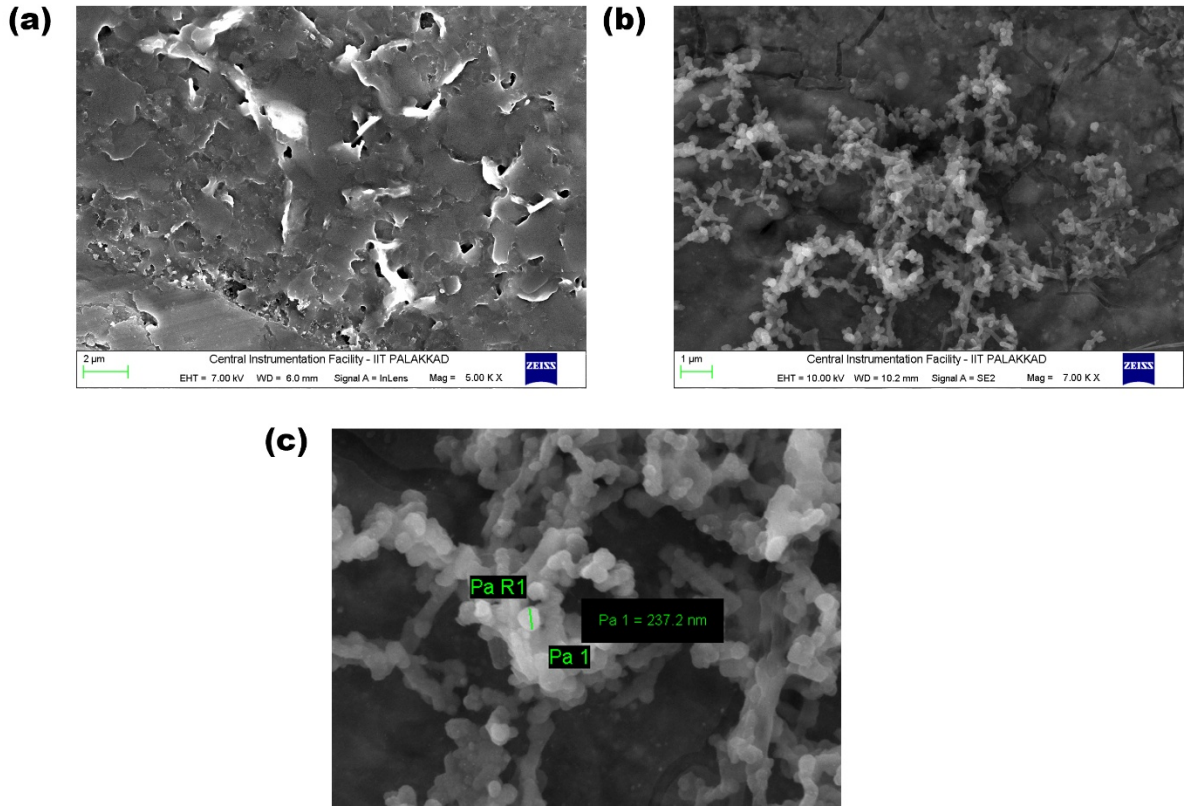


Figure S24. FESEM image of **ATBz** (10 µM) in the (a) absence of cholesterol (b) and (c) presence of cholesterol (50µM).

8.11 NMR titration of ATBz with cholesterol

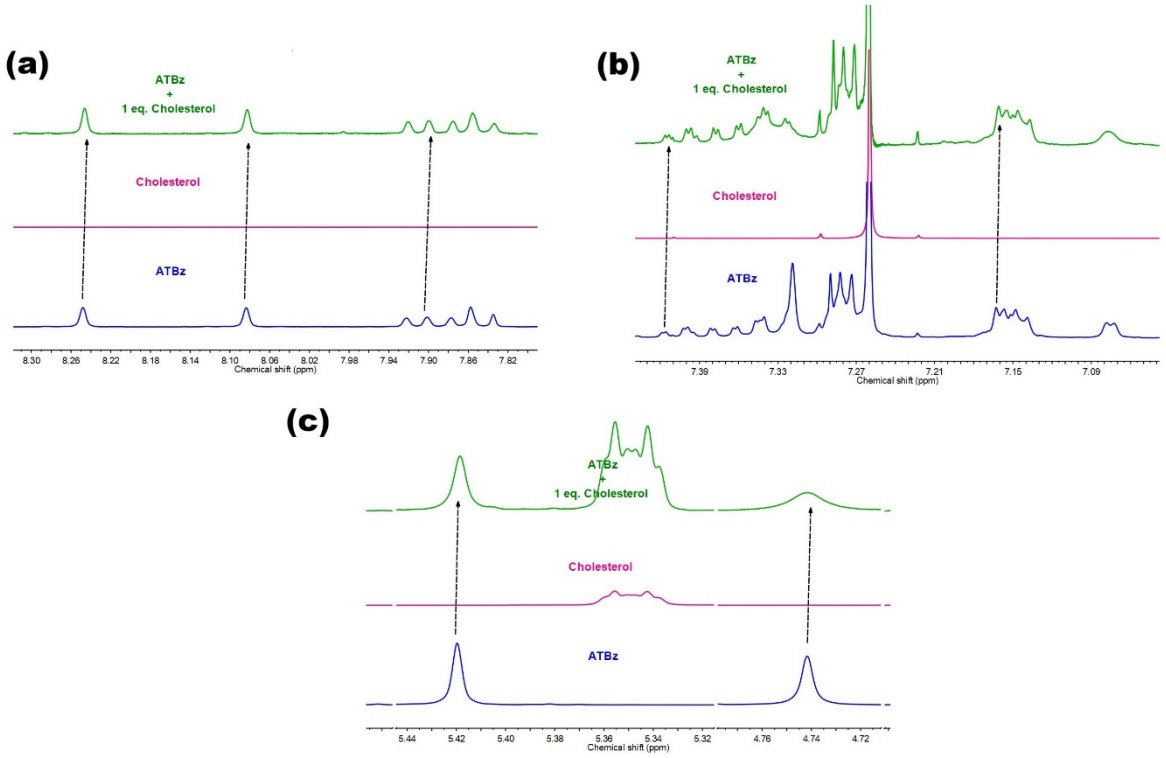


Figure S25. ^{1}H NMR spectrum of **ATBz** in $CDCl_3$ upon the addition of cholesterol.

8.12 PXRD analysis

For PXRD analysis, 10 mg of **ATBz** was dissolved in water containing 1% DMSO and stirred at room temperature for 4-6 hours. The aqueous phase was then allowed to evaporate slowly, after which the resulting precipitate was collected by filtration and dried. To investigate the effect of cholesterol, 1 equivalent of cholesterol was added to the ATBz solution (water with 1% DMSO) and the mixture was stirred under the same conditions for 4-6 hours. Following slow evaporation of the solvent, the obtained solid was filtered, dried, and subsequently analysed by PXRD.

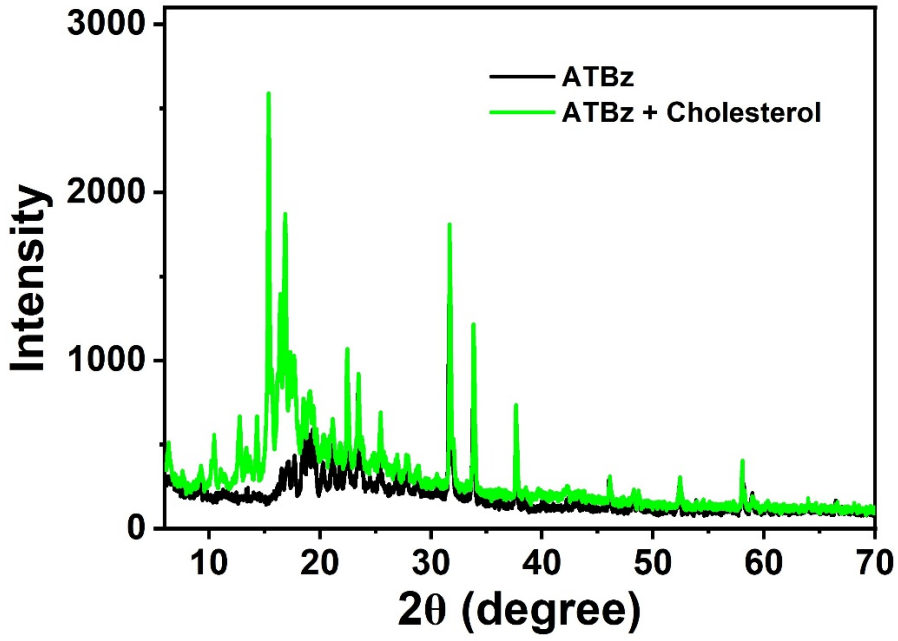


Figure S26. The PXRD analysis of **ATBz** alone (Black) and upon the addition of cholesterol (Green).

8.13 Molecular docking studies

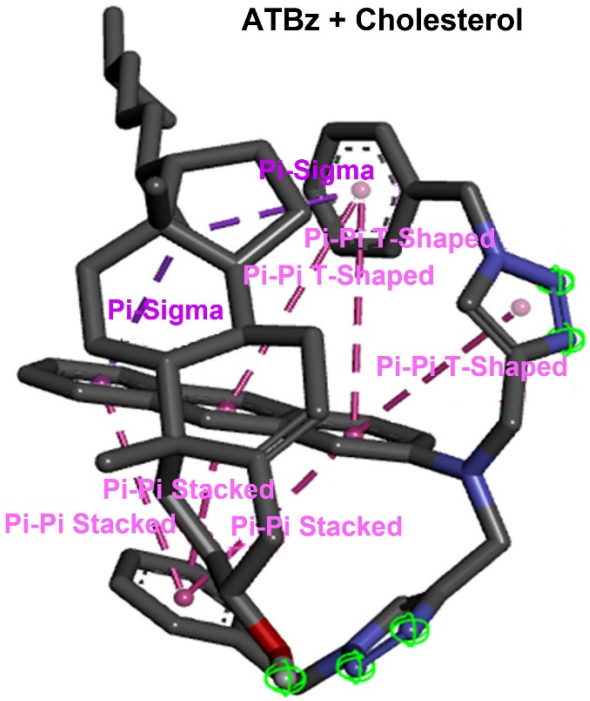


Figure S27. Molecular docking images of **ATBz** and cholesterol visualized using PyMOL and Discovery studio.